METHODS FOR ASSESSING MUSCLE PROTEIN WASTING
AND THERAPEUTICS THEREFOR

CROSS REFERENCE TO RELATED APPLICATIONS

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BACKGROUND OF THE INVENTION

The field of this invention is medicine, and in particular, this invention relates to assays for muscle protein degradation (wasting), to assays to monitor the efficacy of a treatment for wasting in patients and to the use of assays for actin degradation products.

Because of the detrimental effects of wasting, especially of muscle, which is associated with variety of catabolic disorders, there is a need in the art to rapidly diagnose wasting in a patient before the damage is extensive and debilitating so that appropriate treatments can be administered. There is also a longfelt need in the art for ways to identify therapeutic compositions which prevent or reduce the degradation of muscle proteins associated with wasting. The present invention meets these needs.

SUMMARY OF THE INVENTION

The present invention provides methods for the diagnosis of muscle protein wasting and for monitoring the efficacy of treatments for inhibition or prevention of muscle protein wasting in a patient suffering from or susceptible to muscle protein wasting. Specifically, the present inventors have identified a particular actin breakdown product (of about 14-15 kDa) as characteristic of the CASPASE 3-mediated breakdown of muscle protein associated with wasting.

In diagnostic or treatment monitoring methods, a muscle biopsy specimen is obtained from the patient, homogenized, the proteins are reduced and size-separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the actin-related proteins are detected using immunoblotting techniques. Desirably the specimen (sample) is from about 5 to about 500 mg, typically about 50 mg. A protein of about 14-15 kDa which cross reacts with antibody specific for actin is a marker of muscle protein wasting. When a patient being tested for muscle protein wasting displays the actin-related degradation product of about 14-15 kDa in muscle biopsy tissue, then that patient is deemed to be undergoing muscle protein wasting.

If a patient who has been exhibiting symptoms of protein wasting is responding to a treatment designed to inhibiting the wasting, the 14-15 kDa actin degradation product in the muscle biopsy tissue is reduced or is no longer present.

Animal models for muscle protein wasting can be treated with inhibitors of muscle protein wasting (caspase 3 inhibitors). Suitable animal models include, but are not limited to, rats in which diabetes has been induced by streptozotocin. The present invention further provides a method for increasing muscle mass or preventing loss of muscle mass in a subject achieved by administering to the subject an effective amount of a caspase enzyme inhibitor or an inhibitor of an activator of caspase enzyme ord of the enzymes that activate caspases (e.g., enzymes or chemicals that block the activity of phosphatidylinositol 3-kinase). Muscle mass is increased in a normal human or animal, in a human or animal recovering from a muscle wasting condition or in a patient with a catabolic condition. Alternatively, inhibitors of specific caspase enzymes (e.g.

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caspase 3) or inhibitors of the activation of caspase enzymes (e.g., a dominant negative gene or myoblasts transfected to express an inhibitor of caspases) can be introduced locally into muscle. Likewise, a "muscle-specific" gene could be used to avoid a generalized suppression of apoptosis events in other tissues/organs in animals and in the future, patients. A reduction in the amount of the actin degradation product in a muscle biopsy sample is characteristic of a positive response to the treatment. Similar studies can be carried out with muscle cells in culture, with actin proteins and degradation products being resolved using size separation techniques and identified with an actin-specific antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the induction of apoptosis as measured using DNA fragmentation with increasing dosage of staurosporine. DNA fragmentation was analyzed 24 hours after the addition of staurosporine to growing L6 muscle cells.

Figure 2 shows the time-dependent cleavage of actin in lysates incubated for 0-3 hours at 37°C. Lysates were prepared from apoptotic L6 muscle cells (induced for 24 hours with 10 nM staurosporine). Lysate proteins were reduced and solubilized and separated by SDS-PAGE. Actin-related proteins were visualized by Western blotting using antibody specific for the C-terminal 11 amino acids of actin. The positions of the 14 kDa and 42 kDa protein bands are marked.

Figure 3 demonstrates that staurosporine added to growing L6 muscle cells triggers cleavage of actin to yield a 14 kDa degradation product which cross-reacts with actin-specific antibody. The increase in the amount of the 14 kDa protein with increasing dosage of staurosporine is obvious in this image. Proteins were analyzed after 3 hours of incubation at 37°C, with lysates prepared from cells treated for 24 hours with 0-50 nM staurosporine.

Figure 4 shows the activation of caspase 3 with increasing concentrations of staurosporine added to growing L6 muscle cells and incubated for 3 hours. The caspase-specific proteins were

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separated by SDS-PAGE and visualized by immunoblotting with polyclonal antibodies prepared in response to the N-terminus of caspase 3.

Figure 5 illustrates that actin cleavage is mediated by caspase 3. Treatment of the L6 muscle cells with staurosporine and/or the caspase inhibitor Ac-DEVD-CHO is shown.

Figure 6 shows the effects of ATP, staurosporine, temperature and the proteasome inhibitor MG132 on actin degradation. Lysates are prepared and the proteins in the cell lysates incubated for 3 hours at 37°C; lysates were prepared from L6 cells treated with 10 nM staurosporine for 24 hours.

Figure 7 shows the effects of the proteasome inhibitor MG132 (20 μM) on cells treated with staurosporine (10 nM, 24 hours) or on control cells. Actin cleavage was measured in lysates immediately after lysis.

Figure 8 demonstrates that the degradation of the 14 kDa actin degradation fragment involves the N-end rule pathway. Extracts of staurosporine-induced cells were incubated with the dipeptide inhibitor of E3α, Arg-Ala (2 mM) or 2 mM ATP during incubation at 37°C for 3 hours.

Figure 9 demonstrates that overall protein degradation is increased in staurosporine-treated L6 muscle cells. L6 muscle cells were treated as marked, and lysates were incubated at 37°C for 3 hours in the presence or absence of ATP (2mM). Degradation of endogenous protein was monitored by measuring release of acid-soluble tyrosine from cell protein.

Figure 10 demonstrates that the actin fragment is present in a model of accelerated degradation of muscle proteins, namely, streptozotocin-induced diabetes. Muscles from diabetic and sham-injected, pair-fed rats were harvested, homogenized and tested for the presence of the actin fragment. There was more than a two-fold increase in the amount of the 14 kDa actin

fragment in muscles of diabetic rats. Similar findings were obtained in another rat model of a catabolic condition (chronic renal insufficiency) compared to sham-operated, pair-fed rats.

DETAILED DESCRIPTION OF THE INVENTION

5   Loss of lean body mass is a major complication of many catabolic conditions including, but not limited to, uremia, sepsis, cancer, metabolic acidosis and diabetes. Studies of rats and humans with catabolic conditions show that the primary proteolytic pathway stimulated in muscle is the ubiquitin-proteasome pathway.

10   Purified monomeric myofibril proteins are degraded rapidly by the ubiquitin-proteasome pathway. However, intact myofibrils or soluble actomyosin protein complexes are resistant to degradation buy this pathway. These finding indicate that an important step in the proteolysis of the myofibrillar proteins is the dissociation of free myosin, actin and/or other contractile proteins.

15   Signals that activate muscle proteolysis in catabolic conditions could be used to identify the initial step(s) that result in breakdown of muscle proteins. Diabetes activates muscle proteolysis via the ubiquitin-proteasome system and insulin, a well-known inhibitor of apoptosis, rapidly reverses this response. Moreover, chronic renal failure, sepsis, cancer, burn injury, etc. are associated with higher levels of cytokines and cytokines can trigger apoptosis pathways including

20   activation of caspases, a family of aspartic acid proteases (17). For these reasons, we examined whether signals that activate caspase activity in muscle cells could initiate actomyosin dissociation and degradation of protein.

        We found that staurosporine activates apoptosis in cultured L6 muscle cells as determined

25   by the Death ELISA assay (commercially available from Boehringer Mannheim). The induction of apoptosis activates aspartic acid proteases, and we found that staurosporine does induces caspase 3 activity in L6 cells (Fig.4) To determine if caspase 3 can cleave actomyosin, we added recombinant, active caspase 3 to isolated pure actomyosin complexes. Subsequently, proteins were separated and subjected to Western blot analysis using affinity-purified, polyclonal antibodies

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against the carboxy-terminus of actin (Sigma St. Louis, MO). There was a major ⁻42-kDa band that corresponds to the expected size of monomeric rat skeletal muscle actin (Mr = 41,816). In addition, there was a second ~14 kDa band representing an actin fragment of approximately 14 kDa. These results indicate that caspase 3 can cleave actomyosin complexes and actin resulting in fragments of the constituent proteins.

Next, we incubated L6 muscle cells with 50 nM staurosporine for 3 h and then subjected them to lysis, and the resulting cell extracts were incubated at 37° C for 3 to measure overall protein degradation as the release of tyrosine (18,19). With this method, protein degradation in extracts of control cells was slightly less than that in extracts from staurosporine-treated cells. However, the 3 hour incubation at 37° C could limit ATP and impair activity of the ubiquitin-proteasome system. When we added exogenous ATP to the extracts, tyrosine release by extracts from staurosporine-treated cells was significantly higher than by control cell extracts. These results indicate that stimulation of apoptotic pathways accelerate protein degradation in muscle cells.

Since caspase 3 is activated by induction of apoptotic pathways and can cleave actomyosin complexes, we expect that fragments of actin are generated in muscle cells stimulated to activate apoptosis. To test this possibility, L6 cells were treated with 50 nM staurosporine in 2% horse serum for 3 h. The cell extracts were incubated at 37° C for various times before proteins were separated and subjected to Western blot analysis using commercially available, affinity-purified, polyclonal antibodies directed against the carboxy-terminus of actin. Again, there was a major ~42 kDa band that corresponds to the expected size of monomeric rat skeletal muscle actin (Mr = 41,816) in extracts incubated at 37°C. Incubation for 90 minutes resulted in the appearance of a second ~14 kDa band; the intensity of this band increased with longer incubation times.

The appearance of the ~14 kDa actin fragment was dependent upon the amount of staurosporine added to the cells (Fig. 2) with 100 pM staurosporine, the cleaved 14 kDa actin fragment was detected in cell extracts and its amount increased with higher concentrations of

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staurosporine. The 14 kDa actin fragment was absent in extracts from L6 cells kept on ice following treatment with 50 nM staurosporine; thus, we have concluded that the appearance of this fragment is the result of enzymatic action.

5   Stimulation of the apoptotic pathways results in a proteolytic cascade with activation of several isoforms of caspase. Since we found that staurosporine induces caspase 3 activity in L6 cells, we examined whether caspase 3 is involved in the appearance of the 14 kDa actin fragment using an inhibitor of caspase 3. The inhibitor was designed from the amino acid recognition sequence of caspase 3, DEVD, and a cell-permeable inhibitor derivative, Ac-DEVD-Cho, has

10   been developed (20,21). To induce caspase 3 activity, L6 muscle cells were treated with 10 nM staurosporine or serum starvation, and cell extracts were prepared. The extracts were incubated with or without 5 μM Ac-DEVD-Cho for 3 hr and then tested for the appearance of the 14 kDa actin fragment: Ac-DEVD-Cho blocked the appearance of the actin fragment (Fig. 5). This finding indicates that activation of caspase 3 can be an initial step in the proteolytic processing of

15   actin. To support this conclusion, we incubated lysates of L6 muscle cells at 37°C with recombinant caspase 3 and then used Western blotting and detected the appearance of the actin fragment. The 14 kDa actin fragment was present as it was when we added recombinant, active caspase 3 to isolated pure actomyosin complexes.

20   Interestingly, the 14 kDa actin fragment was not detected in cell lysates that had been incubated for short periods even though we found that caspase 3 is rapidly activated by incubation with staurosporine. Previously, Sun et al. noted that actin fragments are not detected when apoptotic pathways are stimulated in muscle cells. One possible explanation for this result could be a rapid degradation of the actin fragments. For example, ATP could become rate-limiting with

25   prolonged incubations resulting in accumulation of the actin fragments. In this case, adding ATP to cell lysates during the 37°C incubation would eliminate the actin fragments. To test this possibility, 2 mM ATP was added to extracts from staurosporine-treated cells at the start of the 3 hr, 37°C incubation. As seen in Fig.6, the amount of the 14 kDa peptide present was significantly reduced compared to the amount present when the same extract was incubated without

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added ATP. To test for involvement of the proteasome, we added MG132, an inhibitor of proteasome activity (ProScript, Inc. Cambridge, MA), and ATP to cell extracts before the 3 hr incubation at 37°C. Adding MG132 resulted in greater amounts of the 14 kDa actin peptide, despite the presence of ATP (Fig.6).

These *in vitro* results are consistent with a proposed sequence of events beginning with apoptotic signals that generate active caspase 3, which cleaves actomyosin complexes and actin to yield the carboxy-terminal fragment of actin. This fragment (and others) are then rapidly degraded by the ubiquitin-proteasome system. To determine if similar events occur in intact cells, L6 cells were incubated for 24 hr with staurosporine in the presence or absence of 100 mM MG 132; control cells were treated with MG132 alone. The cells were then lysed and tested for the presence of the 14 kDa actin peptide. There was no detectable 14 kDa actin fragment in L6 cells that had been treated with staurosporine alone and there was only a small amount of the 14 kDa actin fragment in cells treated with MG132 alone. However, in L6 cells activated with staurosporine but treated with the proteasome inhibitor, the actin peptide was readily detected (Fig.5).

Recently, it was reported that degradation of muscle proteins by the ubiquitin-proteasome system in catabolic conditions involves a combination of a specific E2 ubiquitin-carrier protein and E3 ubiquitin-protein ligase and involved the N-end rule pathway (22,23). The strategy involved blocking proteolysis with the dipeptide, Lys-Ala, which inhibits E3a ligase activity. To determine how activation of caspase 3 influences the N-end rule pathway, L6 cell extracts were prepared following exposure to staurosporine and incubated at 37 °C with or without the dipeptide Arg-Ala and ATP. As with the results of adding the proteasome inhibitor, MG132, the presence of this inhibitor of E3α activity resulted in a greater amount of the 14 kDa actin fragment compared to cells incubated with ATP alone. These results indicate that the N-end rule pathway is involved in degradation of the 14 kDa actin fragment.

Physiological conditions trigger caspase 3-mediated actin cleavage . To examine whether a catabolic condition that is known to accelerate protein degradation in muscle cells also involves actin cleavage, L6 muscle cells were activated by 24 h of serum starvation (0.5% FBS) and cell lysates were prepared. Serum starvation resulted in actin cleavage. This actin cleavage is caspase 3-dependent because adding Ac-DEVD-Cho to the cell lysate during the incubation blocked the accumulation of the 14 kDa actin fragment. Additional evidence for involvement of apoptosis-associated pathways was obtained by activating L6 cells using the serum starvation procedure and then adding insulin or IGF I because either hormone can prevent serum starvation induced apoptosis (24,25). Addition of 10 nM insulin or IGF I effectively blocked serum starvation-induced actin cleavage.

To determine if these results obtained in cultured cells also occur in intact animals, we examined the muscle of rats with acute diabetes, a condition that causes loss of muscle mass by accelerated protein degradation via activation of the ubiquitin-proteasome system (4,26). Rats were treated with streptozotocin and compared to pair-fed, rats injected with saline as described (4,26). Crude muscle lysates were isolated from acutely diabetic and control rats and examined for the presence of the 14 kDa actin fragment. A significant amount of this actin fragment was present in the muscle of diabetic rats compared to results from control rats. Finally, epitrochlearis muscles were incubated in Krebs-Henseleit bicarbonate buffer with or without the addition of the cell-permeable inhibitor of caspase 3, Ac-DEVD-Cho. Incubation with this inhibitor reduced the rate of muscle protein degradation by 30%.

To determine if a condition that activates protein degradation in muscle also activates actin degradation, we studied rats with streptozotocin-induced diabetes (4,26) and their sham-injected, pair-fed controls. This diabetic animal model exhibits accelerated loss of muscle mass and protein degradation in muscle that is mediated by the ubiquitin-proteasome pathway, and there was a more than two-fold increase in the actin fragment in muscle of the diabetic rats. This response is consistent with the results showing that caspase 3-induced degradation of actin leads to its degradation by the ubiquitin-proteasome system.

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Monoclonal or polyclonal antibodies, preferably monoclonal, specifically reacting with a protein of interest can be made by methods well known in the art. See, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratories; Goding (1986) *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, New York; and Ausubel

5   et al. (1993) *Current Protocols in Molecular Biology*, Wiley Interscience/Greene Publishing, New York, NY.

Antibodies specific for Arg-gingipains may be useful, for example, as probes for screening DNA expression libraries or for detecting the presence of Arg-gingipains in a test sample.

10   Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or noncovalently, a substance which provides a detectable signal. Suitable labels include but are not limited to radionuclides, enzymes (including but not limited to, alkaline phosphatase and horse radish peroxidase), substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. United States Patents describing the use of such labels include but

15   are not limited to Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Antibodies specific for an actin degradation product, especially a C-terminus degradation product, are useful identifying proteolytic breakdown products of actin resulting from the

20   activation of caspase 3. Such antibodies can be obtained by the methods well known to the art or they can be purchased from commercial suppliers, for example, Sigma Chemical Co., St. Louis, MO.

The discussion of the invention given herein is provided for illustrative purposes, and is

25   not intended to limit the scope of the invention as claimed herein. Any variations in the exemplified articles which occur to the skilled artisan are intended to fall within the scope of the present invention.

All references cited herein are incorporated in the present application to the extent that there is no inconsistency with the present disclosure.

EXAMPLES

5   Example 1. Cell Culture and Culture Conditions

L6 skeletal muscle cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM medium (1000 mg/l glucose) containing 10% het-inactivated fetal calf serum under humidified 5% CO_2-95% O_2 atmosphere at 37°C. Confluent cells were induced to undergo apoptosis by adding staurosporine to a final concentration of 10 nM. Apoptosis was assessed with

10   the Death-ELISA kit (Boehringer Mannheim, Indianapolis, IN).

Example 2. Cell Extract Preparation

L6 muscles cells were washed and collected in ice cold PBS. Cells were swelled in hypotonic buffer (5 mM Tris pH 8.0, 1 mM 2-mercaptoethanol, 1% glycerol, 1 mM EDTA, 1

15   mM EGTA containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), leupeptin (5 μg/ml) and aprotinin (5 μg/ml) on ice for 30 minutes, and the cells were then gently homogenized with twenty strokes of a dounce homogenizer, with the materials being kept on ice. Homogenates were centrifuged at 3500 rpm (about 2000 x g) for 10 minutes to remove nuclei and cell debris.

20   Example 3.   Assessment of Actin Degradation

To assess for proteolytic cleavage of actin, extracts (20 ug of total protein) were incubated in 20 μl reaction volumes containing 25 mM Hepes (pH 7.5), 5 mM EDTA, 5 mM DTT for 3 h at 37 ° C. Reactions were terminated by adding Laemmli sample buffer, proteins were separated by SDS-PAGE, and immunoblot detection of actin fragments was carried out with an anti-actin

25   antibody (Sigma).

To examine for the activation of actin degradation in a model expressing high activity of muscle protein degradation linked to activation of the ubiquitin-proteasome pathway, we examined the muscle of rats with streptozotocin-induced diabetes and sham-injected, pair-fed, control rats

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(26). Gastrocnemius muscles were homogenized in the hypotonic buffer (5mM Tris (pH 8.0), 1mM 2-mercaptoethanol, 1% glycerol, 1mM EDTA and 1mM EGTA containing 0.1 mM phenylmethanesulfonyl-fluoride (PMSF), leupeptin (5 ug/ml), and aprotinin (5ug/ml)) on ice using 20 strokes of a dounce homogenizer. The homogenates centrifuged at 3500 rpm for 10 min to

5   remove nuclei and debris and the pellet was resuspended in Laemmli-sample buffer before boiling for 5 minutes. The samples were centrifuged at 15,000 rpm (about 10,000 x g) and proteins in the supernatant were separated by SDS-PAGE before an immunoblot of actin fragments was carried out with an anti-actin antibody (Sigma).

10   Example 4. Immunodetection.

After homogenate samples were reduced and solubilized in sample buffer, the proteins were size-separated using SDS-PAGE (14% polyacrylamide gels). The proteins were then transferred to ECL hybond membranes, and blots were incubated with anti-actin antibodies (1:500 dilution) in 5% milk in TTBS for 2 hours at 37°C. Bound primary antibodies were detected with

15   goat anti-rabbit IgG antibody conjugated with HRRP and analyzed with ECL Detection Kit.

Example 5. Degradation of Endogenous Proteins.

Protein degradation in cell extracts was assessed by measuring the net release of free tyrosine because muscles neither synthesizes nor degrades this amino acid (18,19). For 3 hours,

20   50 μg of cell extract proteins were incubated at 37 °C in 0.1 ml of reaction buffer (20 mM Tris (pH 7.6), 2 mM DTT, 10 mM MgCl$_2$, 100 mM KCl, 5% glycerol) with or without 2 mM ATP. Trichloroacetic acid (10% final concentration) was added and the amount of free tyrosine released from proteins was measured spectrofluorometrically. Alternatively, total cell protein can be labeled by growth in the presence of one or more radioactive amino acids, e.g., ^{14}C-labeled or [3]H-

25   labeled, and solubilization of radioactive label can be measured with the use of liquid scintillation counting.

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